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Regulation of immunoreactive-insulin release from a rat cell line (RINm5F)

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1. An insulin-producing cell line, RINm5F, derived from a rat insulinoma was studied. 2. The cellular content of immunoreactive insulin was 0.19 pg/cell, which represents approx. 1% of the insulin content of native rat β-cells, whereas that of immunoreactive glucagon and somatostatin was five to six orders of magnitude less than that of native α- or δ-cells respectively. 3. RINm5F cells released 7–12% of their cellular immunoreactive-insulin content at 2.8 mM-glucose during 60 min in Krebs–Ringer bicarbonate buffer. 4. Glucose utilization was increased by raising glucose from 2.8 to 16.7 mM. There was, however, no stimulation of immunoreactive-insulin release even when glucose was increased from 2.8 to 33.4 mM. A small stimulation of release was, however, found when glucose was raised from 0 to 2.8 mM. 5. Glyceraldehyde stimulated the release of immunoreactive insulin in a dose-dependent manner. 6. At 20 mM, leucine or arginine stimulated release at 2.8 mM-glucose. 7. Raising intracellular cyclic AMP by glucagon or 3-isobutyl-1-methylxanthine stimulated release at 2.8 mM-glucose with no additional stimulation at 16.7 mM-glucose. 8. Stimulation of immunoreactive-insulin release by K^+ was dose-related between 2 and 30 mM. Another depolarizing agent, ouabain, also stimulated release. 9. Adrenaline (epinephrine) inhibited both basal (2.8 mM-glucose) release and that stimulated by 30 mM-K^+. 10. Raising Ca^{2+} from 1 to 3 mM stimulated immunoreactive-insulin release, whereas a decrease from 1 to 0.3 or to 0.1 mM-Ca^{2+} lowered the release. 11. These findings could reflect a relatively specific impairment in glucose handling by RINm5F cells, contrasting with the preserved response to other modulators of insulin release.

The use of isolated islets of Langerhans as an experimental tool has greatly advanced our understanding of the function of the pancreatic β-cell (Hedeskov, 1980; Ashcroft, 1980; Wollheim & Sharp, 1981). However, for the characterization of the mechanism and the regulation of insulin production and release at a more molecular level than has been achieved to date, large quantities of cellular material are required. This is not practical using isolated islets or primary cultures of islet cells, which display only limited proliferative capacity (Rabinovitch *et al.*, 1980). One potentially attractive source of cells for such studies would be an insulin-producing cell line, and, indeed, attempts have been made to obtain such lines by using a variety of techniques (Adcock *et al.*, 1975; Niesor *et al.*, 1979; Santerre *et al.*, 1981; Rae *et al.*, 1979). Clearly, an insulin-producing cell line will only be of value if the cells retain full differentiated function in parallel with their ability to proliferate in culture.

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The present study was undertaken to investigate the functional characteristics of an insulin-producing cell line, RINm5F (Gazdar *et al.*, 1980; Bhathena *et al.*, 1982), derived from a rat islet-cell tumour (Chick *et al.*, 1977). To this end the response of these cells to a variety of agents known to stimulate or inhibit insulin release from native pancreatic β-cells was examined.

Methods

Cell culture

The insulin-producing cell line (RINm5F) used in this study was generously provided by Dr. D. H. Mintz, University of Miami, FL, U.S.A., by courtesy of Dr. A. Gazdar, Georgetown University, Washington, DC, U.S.A. This cloned line was established in culture after a nude-mouse heterotransplant of an X-ray-induced transplantable rat islet tumour (Gazdar *et al.*, 1980; Bhathena *et al.*, 1982).

The cells were grown in plastic culture bottles in RPMI 1640 medium supplemented with 10% (v/v)

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foetal-calf serum, 100 i.u. of penicillin/ml, 100 μg of streptomycin/ml and 0.25 μg of fungizone/ml. Cells were seeded at a density of 2.5×10^4 cells/ml in a total volume of 10 or 20 ml of medium for 25 cm^2 and 75 cm^2 culture bottles respectively. The medium was changed every 3 days and the cells were detached from the bottles with trypsin 1 week after seeding [0.025% trypsin in Ca^{2+}/Mg^{2+}-free physiological saline (0.9% NaCl)/0.27 mM-EDTA, 3–7 min at 37°C]. The trypsin-treated cells were then diluted appropriately for reseeding in culture bottles or in Petri dishes or test plates as described below. The mean population doubling time in exponential growth phase was of the order of 48 h.

Short-term incubations and preparation of cell extracts

For short-term incubation experiments, cells [$(2–5) \times 10^5$ cells] were seeded in either 35 mm-diameter plastic Petri dishes (in 2 ml of medium) or in 16 mm-diameter multi-well test plates (1.5 ml of medium per well) 4 days before the experimental period, and the medium was changed 1 day before the incubation.

On the day of the experiments, the culture medium was aspirated and the cells were washed three times with 1 ml of a modified Krebs–Ringer bicarbonate buffer containing 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], 5 mM-$NaHCO_3$, 5 mg of bovine serum albumin/ml (KRB/Hepes) and 2.8 mM-glucose. Short-term release of immunoreactive insulin was monitored over 30 or 60 min at 37°C in 1 ml of KRB/Hepes supplemented with appropriate agents as noted for each given series of experiments. When the effects of glucagon were studied, the KRB/Hepes (in both the presence and absence of glucagon) was supplemented with Aprotinin (Trasylol) (400 kallikrein-inhibitory units/ml in order to prevent degradation of the hormone. When adrenaline (epinephrine) was used, the KRB/Hepes was similarly supplemented with 1.1 mM-ascorbic acid to limit oxidation of the catecholamine. No untoward effects of either Trasylol or ascorbic acid on immunoreactive-insulin release from RINm5F cells were seen.

At the end of the experimental period, the KRB/Hepes was transferred to test tubes and centrifuged (150 *g*, 10 min, 2–4°C) in order to remove any cells which might have detached from the dishes or wells during the incubation period. Immunoreactive insulin was measured on extracts of the attached cells obtained by the addition of either 1 ml of acetic acid (1 M)/bovine serum albumin (2.5 mg/ml) or 1 ml of acid ethanol (ethanol/water/conc. HCl, 140:57:3, by vol.).

In order to measure the total cellular content of immunoreactive insulin, glucagon and somatostatin, a large number of cells were grown in 25 cm^2 culture bottles. The cells were extracted with 4 ml of acid ethanol and portions of the extracts were either assayed at appropriate dilutions or freeze-dried and reconstituted three-times concentrated in the immunoassay buffer (see below).

Radioimmunoassays and chromatographic characterization of immunoreactive insulin

Insulin, glucagon and somatostatin were measured by radioimmunoassay using the charcoal separation technique (Herbert *et al.*, 1965) as detailed elsewhere (Trimble & Renold, 1981). Rat insulin, pig glucagon and synthetic cyclic somatostatin were used as standards. Guinea-pig anti-(pig insulin) serum was generously given by Dr. Å. Lernmark, Hagedorn Research Laboratory, Gentofte, Denmark. Rabbit anti-(pig glucagon) serum (BR124) was raised in rabbits in our laboratory (Trimble *et al.*, 1982). Rabbit anti-somatostatin serum was generously provided by Dr. F. Jeanrenaud-Rohner, University of Geneva, Geneva, Switzerland.

In order to estimate the apparent molecular weight of immunoreactive insulin extracted from RINm5F cells, portions of cell extracts were subjected to column chromatography. To this end, cell extracts in 1 M-acetic acid/bovine serum albumin (2.5 mg/ml) were neutralized with 1 M-NaOH and then applied to a Sephadex G-50 (Fine grade) column (dimensions 1 cm × 80 cm) and eluted with 0.2 M-glycine/bovine serum albumin (2.5 mg/ml), pH 8.8. The fractions eluted from the column were then taken for radioimmunoassay in order to measure the immunoreactive insulin. The column was characterized by using native pig insulin as a marker.

Glucose utilization

Glucose utilization was measured by monitoring the conversion of [5-^{3}H]glucose to ^3H_2O (Ashcroft *et al.*, 1972). Cells were incubated in 1 ml of KRB/Hepes containing 1 μCi of [5-^{3}H]glucose/ml (stock specific radioactivity 15.6 Ci/mmol) and either 2.8 or 16.7 mM-glucose. After 60 min at 37°C, the buffer was removed and centrifuged as above to remove any detached cells. Portions of the buffer (200 μl) were then taken to measure the production of ^3H_2O during the incubation period, a method described in detail previously (Halban *et al.*, 1980) being used.

Presentation of data

Results are expressed as means ± S.E.M. The statistical significance for differences between groups was determined by Student's *t*-test for unpaired data.

Sources of materials

The materials used in the present study were obtained as follows: RPMI 1640 medium, fungizone,

foetal-calf serum and Hepes (Gibco–Biocult, Paisley, Renfrewshire, Scotland, U.K.), penicillin and streptomycin (Novo Industri A/S, Copenhagen, Denmark), plastic culture bottles (type 3013F and 3024F) and plastic Petri dishes (type 3001F) (Falcon, Oxnard, CA, U.S.A.), multi-well test plates (Linbro type FB-16,24TC, obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.), trypsin (L:250; Difco, Michigan, MI, U.S.A.). Aprotinin (Trasylol, generously given by Professor G. L. Haberland, Bayer AG, Wuppertal, Germany), rat and pig insulin, pig glucagon, ^{125}I-labelled glucagon, ^{125}I-labelled somatostatin (Novo Research Institute, Bagsvaerd, Denmark), cyclic somatostatin (Serono, Freiburg, Germany), *mono-A*^{14}-^{125}I-labelled insulin (generously given by Dr. B. Frank, Eli Lilly and Co., Indianapolis, IN, U.S.A.), Sephadex G-50 (Pharmacia, Zurich, Switzerland), [5-^{3}H]glucose and Biofluor (New England Nuclear Corp., Dreieich, Germany).

Results

Cellular content of immunoreactive insulin, glucagon and somatostatin

In order to attempt to measure the cellular content of glucagon and somatostatin as well as that of insulin, a series of culture flasks containing large numbers of cells [$(1–2)\times 10^7$ cells] were examined. The content of immunoreactive insulin was 191 ± 5 ng/10^6 cells ($n = 10$), and this value was found to be essentially invariable for more than 50 successive tissue-culture transfers. In order to characterize further the immunoreactive insulin, portions of cell extracts were chromatographed on Sephadex G-50. More than 95% of the immunoreactive products applied to the column were recovered in the eluted fractions, of which 11% was eluted in a volume characteristic of pro-insulin and 89% that of insulin. It should, however, be noted that the anti-insulin serum used for radioimmunoassay in this study has not been characterized for its degree of cross-reactivity with rat pro-insulin, since a suitably pure standard of rat pro-insulin is not available. Thus any assessment of the percentage of pro-insulin relative to insulin may prove to be an underestimate if the degree of cross-reactivity of the antiserum is, as would be anticipated, less than 100%. It was, however, in addition found that immunoreactive material extracted from the cells diluted in a linear fashion against a native rat insulin standard in the radioimmunoassay, suggesting that the native hormone is most probably the major product.

The cellular content of immunoreactive glucagon was 28 ± 10 pg/10^6 cells ($n = 10$), the range being 0–76 pg/10^6 cells. For immunoreactive somatostatin, the cellular content was 5.0 ± 2.5 pg/10^6 cells ($n = 9$), the range being 0–18.4 pg/10^6 cells.

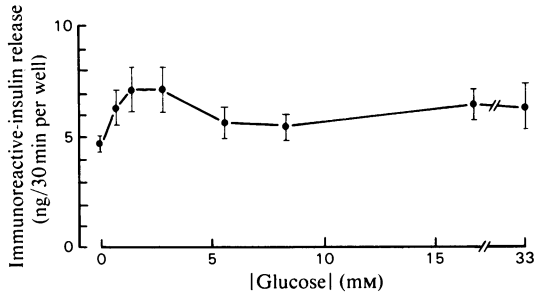

Fig. 1. *Effect of* D-*glucose on release of immunoreactive insulin from RINm5F cells*
RINm5F cells were incubated in multi-well test plates (~10^6 cells/well) for 30 min at 37°C, in 1 ml of KRB/Hepes at the given glucose concentrations. Results are means ± S.E.M. ($n = 12$).

Table 1. *Glucose utilization and release of immunoreactive insulin from RINm5F cells in KRB/Hepes at 2.8 and 16.7 mM-glucose*
Multi-well test plates with ~10^6 cells/well were incubated for 1 h at 37°C. Glucose utilization was determined by measuring the conversion of [5-^{3}H]glucose into ^3H_2O. Results are means ± S.E.M. ($n = 8$); abbreviation used: n.s., not significant.

[Glucose] (mM)	Glucose utilization (nmol/h per well)	P	Immunoreactive-insulin release (ng/h per well)	P
2.8	27.4 ± 2.4	<0.001	12.0 ± 1.0	n.s.
16.7	42.5 ± 2.5		10.6 ± 1.0	

Effects of glucose on short-term release of immunoreactive insulin and on glucose utilization

RINm5F cells were incubated for 30 min with concentrations of glucose ranging from 0 to 33 mM (Fig. 1). There was a stimulation of release of immunoreactive insulin of approx. 50% when the glucose concentration was raised from 0 to 1.4 mM, but no further stimulation was seen at higher glucose concentrations.

In order to determine whether the cells were able to metabolize glucose and to respond to changes in glucose concentration with altered rates of glucose metabolism, glucose utilization was studied in parallel with immunoreactive-insulin release over 60 min. An increase in glucose from 2.8 to 16.7 mM stimulated glucose utilization by 55% without any significant concomitant change in immunoreactive-insulin release (Table 1).

Effects of glyceraldehyde and amino acids on immunoreactive-insulin release

The effects of other known stimulators of insulin release were also studied. Glyceraldehyde has been shown to mimic the effects of glucose on islet

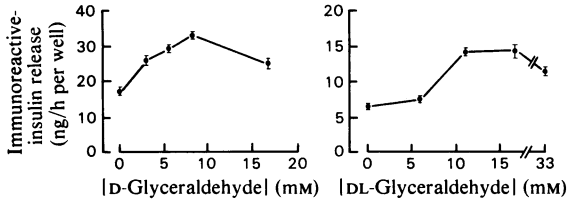

Fig. 2. *Effect of* (*a*) D- *and* (*b*) DL-*glyceraldehyde on release of immunoreactive insulin from RINm5F cells at 2.8 mM-glucose*

RINm5F cells were incubated for 1 h in the presence of 2.8, 5.6, 8.3 and 16.7 mM-D-glyceraldehyde and of 5.6, 11.2, 16.7 and 33 mM-DL-glyceraldehyde. For incubation conditions, see the legend to Fig. 1. Results are means ± S.E.M. [$n = 6$ (*a*); $n = 8$ (*b*)].

Table 2. *Effects of* L-*leucine and* L-*arginine on release of immunoreactive insulin from RINm5F cells at 2.8 mM-glucose*

RINm5F cells were incubated in Petri dishes (~10^6 cells/dish) for 1 h at 37°C in 1 ml of KRB/Hepes with 20 mM-leucine or -arginine. Results are means ± S.E.M. ($n = 17$).

	Immunoreactive-insulin release (ng/h per dish)		
Leucine (20 mM) ...	−	+	−
Arginine (20 mM) ...	−	−	+
	16.2 ± 0.5	25.9 ± 2.3	28.3 ± 2.0

function (Jain *et al.*, 1975). For RINm5F cells it was found that glyceraldehyde (in the presence of 2.8 mM-glucose) stimulated the release of immunoreactive insulin by approx. 100% (Fig. 2). Both D-glyceraldehyde (Fig. 2*a*) and DL-glyceraldehyde (Fig. 2*b*) were tested. The maximal response was similar for the two, whereas the apparent K_m for stimulation of release was lower for the D-form, in keeping with the expected recognition of this racemer by living cells. In both cases, the highest concentration used (16.7 mM for D- and 33 mM for DL-glyceraldehyde) was found to stimulate immunoreactive-insulin release to a lesser extent than the optimal concentration used. In this, as indeed in all other series of experiments, 16.7 mM-glucose failed to stimulate the release of immunoreactive insulin (results not shown).

A comparable degree of stimulation of immunoreactive-insulin release was found when the cells were exposed to 20 mM-L-leucine or 20 mM-L-arginine in the presence of 2.8 mM-glucose (Table 2).

Effects of glucagon and 3-isobutyl-1-methylxanthine on immunoreactive-insulin release

Next, the influence of two substances known to raise islet intracellular cyclic AMP levels was tested.

Table 3. *Effects of glucagon and 3-isobutyl-1-methylxanthine (IBMX) on release of immunoreactive insulin from RINm5F cells in KRB/Hepes*

Multi-well test plates with ~10^6 cells/well were incubated for 1 h at 37°C. Results are means ± S.E.M., and the number of observations is given in parentheses. Level of significance for test versus control at given glucose concentrations: $^*P < 0.001$.

	Immunoreactive-insulin release (ng/h per well)	
[Glucose] (mM) ...	2.8	16.7
Series		
I Control	15.5 ± 1.0 (30)	14.7 ± 0.5 (30)
Glucagon (1 μg/ml)	21.0 ± 1.0 (30)*	22.5 ± 1.0 (30)*
II Control	15.2 ± 0.6 (12)	15.5 ± 0.8 (12)
IBMX (1 mM)	33.2 ± 1.4 (12)*	34.2 ± 3.3 (12)*

Both glucagon (1 μg/ml, 0.33 μM; Table 3, Series I) and 3-isobutyl-1-methylxanthine (1 mM, Table 3, Series II) stimulated immunoreactive-insulin release by 50 to 100%. No additional stimulatory effect of either substance was observed when glucose was raised from 2.8 to 16.7 mM.

Effects of K^+, ouabain and Ca^{2+} on immunoreactive-insulin release

It is well-established that islet cells are extremely sensitive to changes in their cationic environment (Wollheim & Sharp, 1981). For RINm5F cells, the effects of two cations (K^+ and Ca^{2+}) were studied. In both cases, lower and higher concentrations of the ions were used than those encountered in the standard KRB/Hepes buffer (namely 6 mM-K^+ and 1 mM-Ca^{2+}).

At 2.8 mM-glucose there was no significant stimulation of immunoreactive-insulin release when K^+ was raised from 2 to 4 mM (Fig. 3). However, a stimulation of release was found upon increasing K^+ in turn from 4 to 6 mM and from 6 to 15 mM. The effects of lowering or increasing K^+ were also tested in paired experiments at 16.7 mM-glucose. Whereas the sensitivity to increasing K^+ was comparable with that seen at 2.8 mM-glucose (Fig. 3), it was found that 16.7 mM-glucose elicited a small, but significant ($P < 0.005$), stimulation of immunoreactive-insulin release at 2 mM-K^+ compared with 2.8 mM-glucose at the same low ambient K^+. Another substance known, like K^+, to depolarize and stimulate insulin release from islets is ouabain (Siegel *et al.*, 1980). When tested at 2.8 mM-glucose, 1 mM-ouabain increased immunoreactive-insulin release from 9.9 ± 0.8 to 18.0 ± 0.9 ng/60 min per well ($n = 12$).

A decrease in Ca^{2+} (at 2.8 mM-glucose) from 1 to 0.3 mM resulted in a significant ($P < 0.01$) decrease in

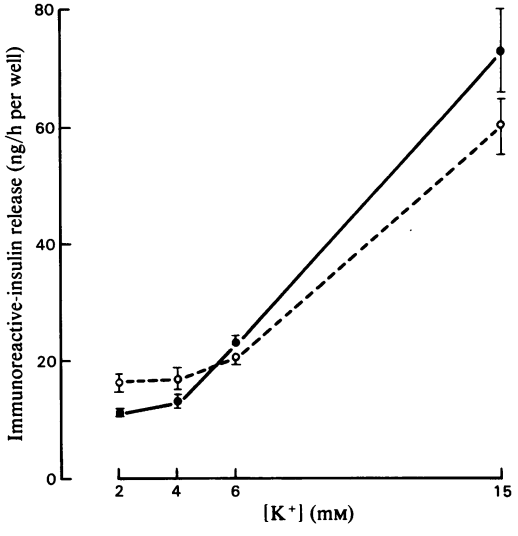

Fig. 3. *Effect of K^+ on release of immunoreactive insulin from RINm5F cells at 2.8 and 16.7 mM-glucose*
RINm5F cells were incubated for 1 h at 2.8 (●) or 16.7 (○) mM-glucose at the given K^+ concentrations. For incubation conditions, see the legend to Fig. 1. The results are means ± S.E.M. ($n = 20$).

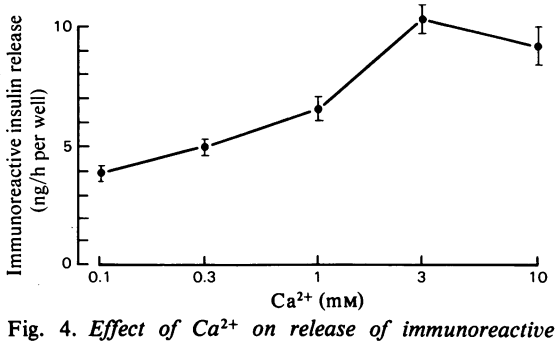

Fig. 4. *Effect of Ca^{2+} on release of immunoreactive insulin from RINm5F cells at 2.8 mM-glucose*
RINm5F cells were incubated for 1 h (as indicated in the legend to Fig. 1) at the given Ca^{2+} concentrations (note the logarithmic scale). Results are means ± S.E.M. ($n = 11$–12).

Table 4. *Effects of L-adrenaline and of K^+ on release of immunoreactive insulin from RINm5F cells at 2.8 mM-glucose*
RINm5F cells were incubated for 1 h (as indicated in the legend to Table 2) with the addition of either 1 μM-adrenaline or 30 mM-K^+ or the contribution of the two. Results are means ± S.E.M. ($n = 11$).

	Immunoreactive-insulin release (ng/h per dish)			
Adrenaline (1 μM) ...	−	+	−	+
K^+ (30 mM) ...	−	−	+	+
	14.0 ± 1.1	5.7 ± 0.3	69.9 ± 3.6	21.2 ± 1.5

immunoreactive-insulin release (Fig. 4), and a further decrease ($P < 0.01$) occurred when Ca^{2+} was lowered from 0.3 to 0.1 mM. An increase in Ca^{2+} from 1 to 3 mM stimulated immunoreactive-insulin release by more than 50%, but no further change in release was seen when Ca^{2+} was raised to 10 mM.

Effects of adrenaline on immunoreactive-insulin release

Finally, having established that the release of immunoreactive insulin from RINm5F cells can be modulated by a variety of agents, the effects of a physiological inhibitor of insulin release, adrenaline, were evaluated (Table 4). Adrenaline (1 μM) inhibited release of immunoreactive insulin at 2.8 mM-glucose by approx. 50%. A significant, but less marked, inhibition of release was observed with 10 nM-adrenaline (result not shown). When release was stimulated by K^+, adrenaline caused a marked reduction in release. However, even in the presence of adrenaline, K^+ still stimulated release, albeit to a lesser extent than that observed in the absence of the catecholamine (Table 4).

Discussion

Previous attempts to obtain insulin-producing cell lines have met with limited success. Thus, when cells from human insulinomas are established in culture, there is most commonly a time-dependent decrease in cellular insulin content, the cells usually becoming completely de-differentiated after several culture passages (Yip & Schimmer, 1973; Adcock *et al.*, 1975). An alternative approach has been to infect pancreatic β-cells with Simian virus 40. In our hands, such Simian-virus-40 transformation of neonatal-rat β-cells did not produce a suitably differentiated cell line (Niesor *et al.*, 1979). More recently, it has been reported that Simian-virus-40 infection of hamster β-cells resulted in cell lines with retained functional capacity, but the cellular insulin content of these cells decreased with time (Santerre *et al.*, 1981). The RINm5F cells used in the present study were originally derived from a rat insulinoma and have been partially characterized previously. It has thus been shown in the original paper (Gazdar *et al.*, 1980) describing several sublines related to RINm5F, that insulin production fluctuated with time in culture, whereas after cloning, lines with relatively enriched levels of either insulin or somatostatin were obtained (Bhathena *et al.*, 1982). We found that RINm5F cells contain levels of immunoreactive insulin which remain constant over more than 1 year in culture. The cellular content of immunoreactive insulin is, however, only 0.19 pg/cell. For native β-cells, it can be calculated, taking the insulin content of adult rat islets (Trimble & Renold, 1981) and the relative number of β-cells in

such islets (Baetens *et al.*, 1979), that the insulin content is 22pg/β-cell. RINm5F cells thus contain approx. 1% of the immunoreactive-insulin content of native rat β-cells.

It has been reported by others (Bhathena *et al.*, 1980, 1982) that RINm5F cells also contain immunoreactive glucagon and somatostatin. By using a calculation similar to that applied to insulin, it can be shown that RINm5F cells contain five to six orders of magnitude less glucagon or somatostatin than do native α- or δ-cells. Indeed, even these very low values may prove to be an overestimate, since the absolute values measured [even when $(1-2) \times 10^{-7}$ cells were extracted] proved variable from one culture flask to the next, some flasks revealing no measurable glucagon or somatostatin. It should be noted that no such variability was found for immunoreactive-insulin cellular content when measured in parallel. These exquisitely low levels of glucagon or somatostatin, taken with the described variability, beg the question as to the significance of these findings. If correct, however, these data could indicate that RINm5F cell cultures contain small numbers of cells producing other islet hormones.

Throughout the present paper we refer exclusively to insulin as determined by radioimmunoassay ('immunoreactive insulin'). It has been shown that immunoreactive material extracted from RINm5F cells has a molecular size by chromatography consistent with that of native proinsulin and insulin and, furthermore, the immunoreactive product(s) dilute in a linear fashion relative to native rat insulin in the radioimmunoassay. It cannot yet be completely excluded that the immunoreactive material studied is a cell product closely related to, but not identical with, insulin.

The main purpose of the present study was to examine the regulation of insulin release from RINm5F cells. Although immunoreactive insulin release was stimulated to a limited extent by glucose (only under certain conditions), this effect was quite different from that with normal pancreatic β-cell preparations [for reviews, see Hedeskov (1980) and Wollheim & Sharp (1981)]. Thus release was only stimulated by 50% and this stimulation arose at very low glucose concentrations (in contrast with native β-cells, which display an apparent K_m for glucose stimulation of insulin release in the range of 8mM). Whereas there was no stimulation of immunoreactive-insulin release between 2.8 and 16.7mM-glucose, glucose utilization by RINm5F cells was increased by 55%, suggesting that these cells are not glucose-insensitive, even in this concentration range. In contrast with glucose, the triose, glyceraldehyde, stimulated immunoreactive insulin in a dose-related manner, and the sensitivity of RINm5F cells is comparable with that found in rat islets (Jain *et al.*, 1975), albeit with reduced responsitivity in terms of maximal stimulation of release. One possible explanation for this could be the high rate of immunoreactive-insulin release under basal conditions (2.8mM-glucose alone), which amounts to 7–12% of the cellular immunoreactive-insulin content/h. This value is considerably higher than that found in normal β-cell preparations (Halban *et al.*, 1982), and this is perhaps consistent with the observation that adrenaline inhibits basal release from RINm5F cells, but not from another preparation of cultured cells, monolayer cultures of newborn-rat pancreatic cells (Marliss *et al.*, 1973). It is currently thought that phosphorylation of glucose by β-cell glucokinase is the rate-limiting step for glycolysis, and, in turn, glucose stimulated insulin release (Hedeskov, 1980; Trus *et al.*, 1981; Trueheart-Burch *et al.*, 1981). The present data on RINm5F cells could imply a defect at this step, when it is recalled that glucose utilization by islet cells has been shown to correlate well with the rate of glucose phosphorylation (Trus *et al.*, 1981). If such a defect is indeed responsible for the lack of glucose sensitivity of RINm5F cells, then substrates which act distal to glucose phosphorylation would be expected to stimulate insulin release. This was found for glyceraldehyde (as discussed above), and for a substance of physiological importance, leucine. It should be noted that the rat islet-cell tumour from which the RINm5F cells originate (Chick *et al.*, 1977) shows increased immunoreactive-insulin release in response not only to leucine (Sopwith *et al.*, 1981) but also to glucose (Masiello *et al.*, 1982). A further interesting facet of the control of insulin production is the observation that, in the rat, only one of the two insulin-gene products is subject to glucose control (Kakita *et al.*, 1982*a*); it will thus be important in future studies to analyse the ratio of rat insulins I and II (Kakita *et al.*, 1982*b*) in RINm5F cells under various conditions.

It is noteworthy that there are other situations where glucose elicits only a poor stimulation of insulin release, whereas glyceraldehyde is found to be fully active. For islets of newborn animals (Ågren *et al.*, 1976) an increase in glucose from 0 to 1.4mM stimulated insulin release, whereas no further stimulation was found at higher glucose concentrations. These data are strikingly similar to those for RINm5F cells presented here. In addition, islets from the newborn animals responded well to glyceraldehyde. The other cases where only a poor response to glucose is observed in the face of retained sensitivity towards glyceraldehyde are in islets from starved animals (Lipson *et al.*, 1979) and from New Zealand obese (NZO) mice (Larkins *et al.*, 1980). Since in these three cases cyclic AMP partially restored glucose sensitivity, this phenomenon was tested in RINm5F cells. Both glucagon and 3-isobutyl-1-methylxanthine (two agents known

to raise intracellular cyclic AMP levels) were found to stimulate immunoreactive-insulin release from RINm5F cells, but there was no difference in release between 2.8 and 16.7mM-glucose, even in the presence of these substances.

An increased concentration of cytosolic Ca^{2+} in the β-cell is thought to trigger insulin release (Wollheim & Sharp, 1981). A simple demonstration of this is to depolarize cells with elevated K^+ which, in turn, results in Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (Atwater *et al.*, 1981; Wollheim *et al.*, 1980). Raising K^+ from 6mM to 15 or 30mM caused a marked increase in immunoreactive insulin release from RINm5F cells. Indeed, this degree of stimulation was higher than that for any other condition used. For these cells, the membrane potential would seem to be an important factor in the regulation of release, since a lowering of K^+ from 6 to 2mM lowered immunoreactive-insulin release. Furthermore, at 2mM-K^+, 16.7mM-glucose caused a small stimulation of release not observed at higher K^+ concentrations.

In normal pancreatic β-cells, Ca^{2+} alone can only induce insulin release under certain conditions, e.g. pretreatment with extremely low Ca^{2+} followed by very high Ca^{2+} (for review, see Wollheim & Sharp, 1981). Strikingly, for RINm5F cells, raising Ca^{2+} from 1 to 3mM stimulated immunoreactive-insulin release. A further difference between β-cells and RINm5F cells was the reduction in release when Ca^{2+} was lowered to 0.3 and 0.1mM. Such a reduction in insulin release from normal islet β-cells would only be expected under stimulatory conditions, but not at 2.8mM-glucose, as used here, and indeed the sensitivity of RINm5F cells to Ca^{2+} recalls that of human insulinomas (Kaplan *et al.*, 1979) rather than that of native β-cells.

In conclusion, RINm5F cells, although displaying a number of differentiated features typical of native β-cells, appear to suffer from certain aberrations in their regulation of immunoreactive-insulin release. The study of these changes may provide further insight into the regulation of insulin release normally occurring in β-cells.

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